

# EXHIBIT B

# Virus interference. I. The interferon

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During a study of the interference produced by heat-inactivated influenza virus with the growth of live virus in fragments of chick chorio-allantoic membrane it was found that following incubation of heated virus with membrane a new factor was released. This factor, recognized by its ability to induce interference in fresh pieces of chorio-allantoic membrane, was called interferon. Following a lag phase interferon was first detected in the membranes after 3 h incubation and thereafter it was released into the surrounding fluid.

## INTRODUCTION

One of the most useful situations for studying interference among animal viruses has been the interference produced by inactivated influenza viruses with the growth of live influenza virus in the chorio-allantoic membrane of the chick embryo. In this system, a number of variables have been measured, e.g. the effects of varying the dose of interfering and challenge virus or the time interval between the two inoculations, the effects of different methods of virus inactivation and the use of different virus strains (see review by Henle 1950). As a result of studies by different workers, it is generally agreed that interference cannot be explained by blockage of cell surface receptors. Fazekas de St Groth, Isaacs & Edney (1952) found that interference by influenza virus inactivated at 56°C took some hours until it was fully established, but it was difficult to decide by experiments in the intact chick embryo whether this time was required for the inactivated virus to be absorbed by the cells or for some further reactions to occur. We have studied this point with pieces of chorio-allantoic membrane suspended in buffered salt solution *in vitro* (Fulton & Armitage 1951; Tyrrell & Tamm 1955) a method which allows observation of fluid and cells separately and manipulations which are not possible in the chick embryo. As a result, a number of new features of the interference reaction have emerged and these are described in this and the following paper.

## METHODS

### *Interfering virus*

The Melbourne (1935) strain of influenza virus A was used as freshly harvested allantoic fluid. It was mixed with a 2% sodium citrate solution in normal saline and borate buffer, pH 8.5, in the ratio 6 parts virus, 2 parts citrate-saline and 1 part borate buffer, and heated at 56°C for 1 h. This treatment abolishes the infectivity and enzymic activity of the virus while retaining its interfering activity (Isaacs & Edney 1950a). In the present experiments, it was shown by inoculating

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eggs allantoically that heating had inactivated the virus; this test was included in most, but not in all of the present experiments. The heated virus is referred to as heated MEL.

#### *Buffer*

The buffer used to suspend and wash the pieces of chorio-allantoic membrane was that described by Earle (see Parker 1950).

#### *Chorio-allantoic membrane*

Pieces of chorio-allantoic membrane were removed from 10- or 11-day fertile hen's eggs by a technique similar to that described by Tamm, Folkers & Horsfall (1953). Six or seven pieces were taken from each egg and membranes were pooled from a group of eggs and randomized. In order to find the weight of a piece of membrane, ten pieces were selected at random, drained on filter paper and weighed. The average weight of a piece of membrane was found to be 20 mg.

#### *Interference and challenge*

An interference experiment was carried out in the following way: Six pieces of membrane were placed in  $6 \times \frac{1}{8}$  in. test-tubes, and to each was added 1 ml. of test material. Six other tubes were similarly incubated with 1 ml. buffer. In each case 100 units of penicillin were added per ml. fluid. The tubes were stoppered and placed in a roller drum at 37°C (8 rev/h). After 24 h incubation the membranes were removed, washed in two changes of buffer and put in fresh tubes along with 1 ml. buffer in which MEL virus at a final dilution of  $10^{-3}$  was incorporated. The MEL virus came from a stock of capillary tubes kept at -70°C and a single stock of virus lasted through almost all the experiments. The tubes were placed in the roller drum for a further 48 h at 37°C after which the fluids were titrated individually for their haemagglutinin content.

#### *Haemagglutinin titrations*

Two-fold dilutions (0.25 ml.) of test virus were made in normal saline using automatic pipettes and plastic plates. To each dilution 0.25 ml. of a 0.5% suspension of chick red-blood cells was added, and the cells allowed to settle. That pattern which showed partial agglutination was taken as the end-point, and it was read by interpolation if necessary. One agglutinating dose (a.d.) is defined as the amount of virus present at the partial agglutination end-point. The readings in the tables are given as  $\log_2$ , i.e. tube number of a series of two-fold dilutions. Thus if the end-point of agglutination occurs at a  $1/2$  dilution the reading is taken as  $\log_2 = 1$ . If the end-point of agglutination occurs at a  $1/4$  dilution the reading is taken as  $\log_2 = 2$ . A  $1/1$  dilution end-point has a  $\log_2 = 0$  and material which did not agglutinate red cells at a  $1/1$  dilution was given an arbitrary score of -1.

#### *Assessment of results*

A group of sixty titrations of control materials carried out in a single experiment was analyzed statistically. The logarithms of the haemagglutinin titres of individual fluids were found to occur in an approximately normal distribution. It was

necessary, therefore, to measure geometric mean haemagglutinin titres when comparing two groups of materials. The mean ( $\log_2$ ) titre of the sixty titrations was 6.78 and the variance 0.412 (standard deviation 0.642). If we took samples of six from this population we should expect that 99 % of the sample means would fall in the range  $6.78 \pm 0.67$ . Therefore, if the means of two samples, drawn at random, differ by more than  $0.67 \log_2$  it is likely that they are drawn from different populations. In practice, we have assumed that if an experimental group of six tubes showed a geometric mean titre of one  $\log_2$  unit less than a group of six controls tested at the same time this indicates a slight but significant degree of interference; a difference of two  $\log_2$  units was taken as showing definite interference.

One difficulty was that groups of controls tested on different days showed highly significant differences in titre. This finding appears to be due to the variable sensitivity of the red cells from different fowls to influenza viral haemagglutinin. In order to overcome this difficulty a control group was included in each experiment, and to facilitate comparison between different experiments the results for an experimental group are shown in the table as the proportion (expressed as a percentage) of the geometric mean haemagglutinin titre of the corresponding controls. Thus a difference of one  $\log_2$  unit between experimental and control groups corresponds to a 50 % yield, a difference of two  $\log_2$  units to a 25 % yield, etc. Small arithmetic differences in the percentage yield have therefore a much greater significance at low than at high percentage yields.

## RESULTS

### *Effect of varying the temperature and time of contact of heated virus and cells on the degree of interference*

In preliminary experiments it was found that interference could be induced in pieces of chorio-allantoic membrane in the following way. Heat-inactivated MEL virus was added to the suspending fluid along with a piece of membrane, and this was then incubated in the roller drum for 24 h at 37°C; controls were incubated in buffer. The membranes were then washed, placed in fresh tubes with live MEL virus diluted  $10^{-3}$  in 1 ml. of buffer, and incubated for a further 40 to 48 h at 37°C. Previous treatment of the membranes with heated MEL in this way caused pronounced interference with the growth of live MEL. In this system, 200 to 400 agglutinating doses of heated MEL almost completely suppressed haemagglutinin production by live virus and the effect of different doses of heated MEL can be seen from the information contained in table 4. Tyrrell & Tamm (1955) found that LEE virus heated at 56°C did not cause interference in a similar system; this difference may be due to virus strain variability.

It was soon found that the time interval between the application of interfering and challenge viruses had an influence on the degree of interference. In order to see what was the importance of the time interval, the following experiment was carried out. Pieces of membrane were mixed with a small dose of heated MEL virus (60 a.d.) in order to produce slight interference, and at varying time intervals, groups of membranes were removed, washed thoroughly in buffer, resuspended

in buffer and further incubated either at 2 or 37°C. The total incubation time was 24 h and this was divided between a *primary* incubation period in contact with heated MEL at 37°C and a *secondary* incubation period of the washed membranes at 2 or 37°C. After the secondary incubation, the membranes were again washed and challenged with MEL virus. The results are shown in table 1. The results show that a primary period of 15 min contact between heated MEL and the membrane was sufficient to establish nearly as much interference as a primary period of 24 h at 37°C, provided the secondary incubation was carried out at 37°C. The findings suggest that the heated MEL virus is rapidly adsorbed to the cells, and thereafter that it does not act as an inert blocking agent. The fact that after 4 h *primary*

TABLE 1. EFFECT OF VARYING TIMES AND TEMPERATURES OF INCUBATION ON THE INTERFERING ACTIVITY OF HEATED MEL

interfering virus	primary incubation	secondary incubation	geometric mean HA titre (log <sub>10</sub> )	% of control titre
60 a.d. of heated MEL	15 min at 37°C	24 h at 37°C	3.9	15
60 a.d. of heated MEL	15 min at 37°C	24 h at 2°C	6.7	> 100
60 a.d. of heated MEL	1 h at 37°C	23 h at 37°C	4.0	33
60 a.d. of heated MEL	1 h at 37°C	23 h at 2°C	5.8	94
60 a.d. of heated MEL	4 h at 37°C	20 h at 37°C	2.3	10
60 a.d. of heated MEL	4 h at 37°C	20 h at 2°C	3.6	20
60 a.d. of heated MEL	24 h at 37°C	nil	1.5	3
buffer control	24 h at 37°C	nil	5.6	100
buffer control	24 h at 2°C	nil	5.9	100

incubation there is a slight difference in interference, depending on whether *secondary* incubation is carried out at 2 or 37°C, implies that some active metabolic process in the membrane requiring at least 4 h incubation at 37°C is necessary before interference is fully established. It is difficult to be sure how long this process takes since it might continue during the early stages of growth of the challenge virus, and 4 h is therefore only a minimal figure.

#### *Stability of interfering activity of heated MEL*

Incidental observations had pointed to some instability of the interfering activity of heated MEL during incubation at 37°C. An experiment illustrating this is shown in table 2. Table 2 shows that the interfering activity of heated MEL virus was reduced about ten-fold by incubating it for 24 h at 37°C before adding the membranes. This degree of instability is interesting since Paucker & Henle (1955) found that the infectivity of the PR 8 strain of influenza virus was inactivated at 37°C at the rate of about one log<sub>10</sub> a day.

When an attempt was made to measure the amount of unabsorbed heated MEL virus after varying times of contact with chorio-allantoic membrane, a difficulty was soon encountered. Apparent rapid 'disappearance' of the haemagglutinin was found to be caused by combination of the virus with an inhibitor of agglutination released by the membrane into the surrounding fluid. This could be shown

by incubating pieces of membrane with buffer at 37°C in the roller drum, when after 2 h sufficient inhibitor of agglutination was released by the membrane into the surrounding fluid to block agglutination by an equal volume of heated MEL virus with an agglutinin titre of 100. A similar effect was noted by Schlesinger & Karr (1956) and probably accounts for the difficulty described by Isaacs & Edney (1956b) in removing the inhibitor in allantoic fluid by frequent washing of the allantoic cavity.

TABLE 2. EFFECT OF INCUBATION AT 37°C FOR 24 h ON THE INTERFERING ACTIVITY OF HEATED MEL

dose (a.d.) of interfering virus	incubation period before test	geometric mean HA titre (log <sub>2</sub> )	% of control titre
373	nil—control	0.8	2.3
124	nil—control	1.9	4
41	nil—control	3.9	20
1120	24 h at 37°C	2.3	5
373	24 h at 37°C	5.4	41

TABLE 3. EFFECT OF MEMBRANE INHIBITOR ON INTERFERING ACTIVITY OF HEATED MEL

heated MEL suspended in	incubation period before test	geometric mean HA titre (log <sub>2</sub> )	% of control titre
buffer	nil	0.8	0.9
membrane inhibitor	nil	3.8	7
buffer	4 h at 37°C	2.3	2.4
membrane inhibitor	4 h at 37°C	5.5	22
control without heated MEL	nil	7.7	100

An experiment was carried out to see what effect this membrane inhibitor might have on the interfering activity of heated MEL. A sample of inhibitor was prepared by incubating normal membranes in buffer. Heated MEL virus was then tested for its interfering activity diluted in this membrane extract or in buffer as a control; also, interfering activity was tested by adding the membranes to these reagents at once, or after the two preparations of heated MEL had been incubated for 4 h at 37°C. The results in table 3 show that the membrane extract had a pronounced inhibitory effect on the interfering activity of heated MEL. Also, the degree of instability of the interfering activity of heated MEL during 4 h incubation at 37°C was not affected by the presence or absence of membrane inhibitor.

#### *Residual interfering activity*

These experiments have indicated that the interfering virus is rapidly taken up by the cells, although interference in the cells takes some time to be established. One would expect, too, that in these experiments little interfering activity would remain in the fluid after 24 h contact between heated MEL and the membrane, since any unabsorbed virus would lose interfering potency as a result of inactivation at 37°C and combination with inhibitor. It was surprising, therefore, to find that

after 24 h incubation considerable interfering activity remained in the surrounding fluid. This can be seen from the experiment illustrated in table 4. In this experiment, different amounts of heated MEL were incubated with pieces of membrane for 24 h at 37°C. Membranes and fluids were then separated and the membranes were washed and challenged with MEL virus to assess the degree of *initial* interference. The amount of *residual* interference in the fluids was measured by adding fresh pieces of membrane to the fluids, incubating 24 h at 37°C and challenging with MEL virus in the same way. Clearly the degree of residual interfering activity is only slightly less than the amount of initial interfering activity.

TABLE 4. COMPARISON BETWEEN INITIAL INTERFERING ACTIVITY OF HEATED MEL AND RESIDUAL INTERFERING ACTIVITY AFTER INCUBATION WITH CHORIO-ALLANTOIC MEMBRANE FOR 24 H

interfering activity measured	dose (a.d.) of heated MEL initially present	geometric mean HA titre ( $\log_2$ )	% of control titre
initial	1120	< 0	< 1.3
initial	373	0.8	2.3
initial	124	1.9	4
initial	41	3.9	20
residual	1120	0.75	1.6
residual	373	3.6	12
residual	124	4.7	25
residual	41	5.7	50

In an effort to explain the results of the last experiment the possibility was considered that fresh interfering activity was produced by the membrane. This possibility was confirmed by the following experiment. Heated MEL virus was incubated with pieces of membrane for 2 h at 37°C. The membranes were then thoroughly washed and incubated in fresh buffer at 37°C. It was found that after some hours' incubation at 37°C fresh interfering activity could be detected in the incubating fluid. Some of the properties of this newly released interfering agent are described in the accompanying paper, but we can anticipate meanwhile by saying that the newly released interfering agent is a non-haemagglutinating macromolecular particle which has many different properties from those of heated influenza virus. To distinguish it from the heated influenza virus we have called the newly released interfering agent 'interferon'. It was also found that the membranes which were liberating 'interferon' showed a diminished production of MEL virus on challenge, i.e. establishment of interference was accompanied by liberation of interferon.

*Time of appearance of interferon in the membrane  
and release into the surrounding fluid*

We next studied the appearance of interferon in the membranes and its liberation into the surrounding medium at different time-intervals after inoculating heated MEL virus. The experiment which is illustrated in figure 1 was carried out in the following way:

Pieces of chorio-allantoic membrane were mixed with a large dose of heated MEL (4000 agglutinating doses/membrane piece) and incubated in the roller drum

for 3 h at 37°C. The membranes were then removed, washed thoroughly in buffer, resuspended in fresh test-tubes with 1 ml. of buffer/membrane piece and re-incubated at 37°C. The end of this time was taken as zero hour and at various time intervals thereafter groups of tubes were removed, and the fluids and membranes tested separately for interferon activity. The membranes were pooled in groups of six, ground in a Ten Broeck grinder, suspended in 6 ml. buffer, lightly centrifuged and the supernatant fluid tested by adding six fresh pieces of membrane. (In a control experiment it had been shown that after lightly centrifuging a membrane extract in this way all the interfering activity was present in the supernatant fluid.) The further test of interfering activity of fluids and membrane extracts was carried out by incubating test fluids with fresh pieces of membrane for 18 to 24 h at 37°C, washing the membranes and challenging with MEL virus. The yields of

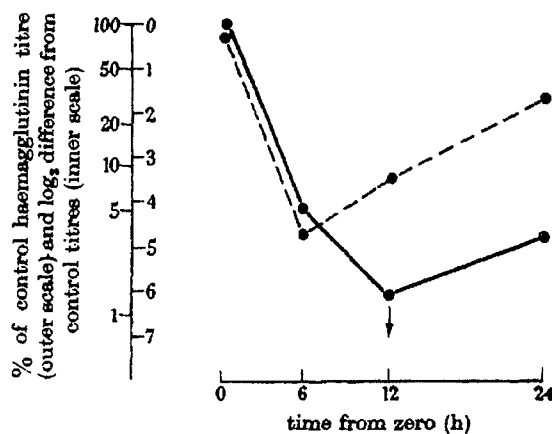


FIGURE 1. Presence of interferon in membranes and fluids.  
— fluids, --- membrane extracts.

haemagglutinin were compared with those of control membranes incubated in buffer. In the experiment illustrated in figure 1, the samples harvested at 0 and 6 h had a different control group from the samples harvested at 12 and 24 h, and in each case the results in the experimental group were compared with their corresponding controls. The fluids and membrane extracts tested at 0 h showed no interfering activity, although the membranes had absorbed large amounts of heated MEL virus, and would themselves probably have been resistant to challenge as shown by control experiments. At 6 h there was a high degree of interferon activity in the membrane extracts and slightly less in the fluids. The greatest interfering activity in the fluids was found at 12 h, a finding which was confirmed in a second experiment, and by this time the activity in the membrane extracts had declined slightly. At 24 h there was little remaining activity in the membrane extracts and most of the interferon had been liberated into the medium.

This type of experiment was repeated with a number of variations. In one experiment an initial period of 2 h contact between heated MEL and membranes was allowed, and samples were taken at 0, 3, 6, 9 and 12 h. In this case, significant



interfering activity was found in membranes and fluids at 3 h, but the potency of both was less than at 6 h. The only difference from the results shown in figure 1 was that in this experiment the zero samples of both membranes and fluids showed slight but significant interfering activity. This finding may have been due to small amounts of heated MEL remaining loosely attached to the membrane; since the same finding was noted when the heated MEL and membranes were left in contact for 24 h at 2°C. Under these conditions, Ishida & Ackermann (1956) have shown that influenza virus becomes loosely and reversibly attached to the chorio-allantoic membrane, irreversible attachment requiring incubation at 37°C. Preliminary incubation of heated MEL and membranes at 2°C followed by washing the membranes did not give satisfactory results and there was a low production of interferons.

In another experiment, the *differential* release of interferons into the fluid was studied. Heated MEL and membranes were kept 2 h in contact at 37°C and the membranes were then washed and incubated in buffer. At two-hourly intervals thereafter the membranes were removed, washed and incubated in fresh buffer; all the manipulations were carried out with warm reagents and at 37°C. The yields of interferon during the different time intervals were then measured and the results are shown in table 5.

TABLE 5. DIFFERENTIAL YIELD OF INTERFERONS AT INTERVALS  
AFTER INOCULATING HEATED MEL

differential sample	geometric mean HA titre (log <sub>2</sub> )	% of control titre
0-2 h	6.7	81
2-4 h	5.3	29
4-6 h	5.2	27
6-8 h	5.7	36
8-10 h	5.8	40
control	6.9	100
10-24 h	7.0	81
control	7.2	100

The maximal liberation of interferon occurred between the second and sixth hours. The yield of interferon was much smaller at each interval, largely due to the fact that the total yield was divided among so many samples. (In the accompanying paper it is shown that interferon prepared as described here has little interfering activity at a dilution of 1 in 10.) In this experiment little interferon was liberated after 10 h, but in another experiment slight activity was found in a 10 to 24 h sample.

*Relationship between interfering activity of the virus and interferon production*

The amount of interferon produced depends on the amount of heated MEL used, as indicated by the results shown in table 4. The following experiment showed that interferon production is also dependent on the possession of interfering activity by the heated MEL. It is based on the fact that MEL virus heated for 1 h at 56°C had strong interfering activity, whereas the same virus heated for 1 h at 60°C had no significant interfering activity (Isaacs & Edney 1950a).

Aliquots of a preparation of MEL virus in citrate-borate buffer were heated for 1 h at 56 and 60°C and incubated with pieces of membrane for 2 h at 37°C. The membranes were then washed and incubated in buffer for a further 20 h at 37°C. Thereafter, the membranes were removed and tested for their ability to support the multiplication of MEL virus, while the fluids were tested for their interferon content. The results are shown in table 6.

TABLE 6. RELATIONSHIP OF INTERFERING ACTIVITY AND INTERFERON PRODUCTION BY MEL HEATED AT 56 AND 60°C

MEL heated at	material tested	geometric mean HA titre (log <sub>2</sub> )	% of control titre
56°C for 1 h	membranes for interference	0.88	1.1
60°C for 1 h	membranes for interference	6.4	53
56°C for 1 h	interferons liberated	2.6	4
60°C for 1 h	interferons liberated	7.1	100

The virus which had been heated at 56°C induced significant interference in the membranes which also liberated interferon. The virus which had been heated at 60°C caused a barely detectable degree of interference and no interferon was liberated.

*Attempted passage of interferon in series*

The results obtained have not made it clear whether interferon is part of the heated MEL virus which is liberated by the membrane, or whether it is newly synthesized in the membrane. In either event it was interesting to test the possibility that interferon might be able to replicate in series. In order to test this, advantage was taken of the fact that interferon exerts its activity if it is left in contact with the membranes for 4 h at 37°C, and the membranes are then washed and incubated in buffer for a further 20 h at 37°C before challenge. Under these conditions no new interferon activity could be detected in ground membranes or in the medium after a single passage or after two serial passages. Controls showed that the interferon grown in eggs or tissue cultures did not produce live virus.

#### DISCUSSION

In earlier studies on virus interference attempts were sometimes made to explain the phenomenon as due to an inert blocking action of the interfering virus preventing the challenge virus from entering the cells. This view has been contested by those who could find no evidence that the interfering virus prevented uptake of challenge virus, and in the present studies it was found that in order to establish interference more than 4 h incubation at 37°C was required. Tyrrell & Tamm (1955) found that incubation at 37°C was necessary for the establishment of interference in a similar system, but using virus 'inactivated' at 22 or 37°C. However, it is known that virus incubated at 22 or 37°C retains some infectivity and is able to undergo a modified cycle of virus multiplication resulting in the production of virus resembling incomplete virus (Henle 1953; Horsfall 1954). In contrast, virus heated at 56°C for 1 h has hitherto shown no evidence of infectivity, or of the

ability to produce virus haemagglutinin or soluble antigens (Isaacs & Fulton 1953). The present results suggest, nevertheless, that interference shows some of the characters we might expect of an abortive attempt at a single cycle of virus multiplication. A second finding which supports this idea (in addition to the fact that interference requires some metabolic activity on the part of the membrane) is that the interfering action of heated MEL is inactivated during incubation at 37°C to approximately the same extent as is the infectivity of unheated virus. But the best support for the idea arises if we consider the interferon provisionally as an abortive product of virus multiplication. This suggestion is made mainly as a guide to further experimentation until the interferon is better characterized chemically and serologically.

The analogies between interferon and virus production are as follows: Little or no interferon activity could be detected in membranes or fluids shortly after inoculating heated MEL; this is analogous to the so-called 'eclipse period', when only a small fraction of the inoculated influenza virus can be recovered from infected cells (Hoyle 1948; Henle 1949). Secondly, the times at which interferon activity can be detected in membranes and fluids correspond very well with those at which new virus antigens appear after inoculating live influenza virus. The fact that interferon is able to inhibit influenza virus growth, but is unable to replicate in series, suggests similarities to the single cycle of viral haemagglutinin production caused by infection with incomplete virus (Burnet, Lind & Stevens 1955; Paucker & Henle 1955).

The experiment which showed that virus heated at 60°C for 1 h had no interfering action and did not lead to the production of interferon, suggests a close relationship between the two phenomena. However, there is insufficient evidence to postulate yet that the influenza viral interference phenomenon is due directly to interferon production. It is also not yet known whether interferon is simply liberated from the heated MEL or is newly synthesized in the membrane. In favour of its being newly synthesized is an observation that in order to obtain good yields of interferon, adequate oxygenation of the membrane is necessary.

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